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Cancer cells can generate constitutively reactive oxygen species (ROS), which are thought to promote cell proliferation, cell motility, invasion and angiogenesis, all of them prerequisites for tumor metastasis. Recently novel ROS-generating enzymes termed Nox have been identified in epithelial cells. Transfer of Nox into non-transformed fibroblasts increased ROS production and rendered these cells tumorigenic. The goal of our project was to identify Nox family members in cancer cells and to evaluate their regulation and cellular function.

Breast cancer cell lines were screened by RT-PCR for the presence of various *nox* and neutrophil NADPH oxidase genes and contained Nox3, Nox4, Nox5 as well as $p22^{phox}$ and $p67^{phox}$. Reactive oxygen production by Nox4 is dependent on the oxidase component $p22^{phox}$, growth factors and cellular adhesion. We observed down-regulation of ROS production when attachment of cells was abolished or under serum withdrawal. In terms of function, Nox4-stimulated ROS generation led to up-regulation of VEGF, a promoter of angiogenesis. The activity of deregulated Nox proteins in cancer cells may have wide ranging implications in tumorigenic events including metastasis.

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INTRODUCTION:

Certain human carcinoma cell lines produce reactive oxygen species (ROS) constitutively. This affords them potentially with an advantage in cell proliferation, and in migration and invasion of surrounding tissues by degrading the surrounding extracellular matrix and by increasing their motility. The basis for generation or upregulation of ROS in cancer cells is so far unknown. We hypothesized that recently identified, novel ROS-producing enzyme systems may be involved in this phenomenon and proposed to define members of this family in breast cancer cells and to evaluate their role in events leading to metastasis.

BODY:

To provide more continuity for this report, we decided to change the order in which Technical Objectives and their results to date are presented. All three objectives have been investigated in parallel and their outcomes depended on each other.

Objective 3: Characterization of the ROS generating enzyme in cancer cells

We have analyzed the identity of the ROS-generating enzyme in cancer cells. According to our hypothesis, we focused our efforts on NADPH oxidase components and cytochrome b_{558} homologs (1-5, 7, 9, 12). We reported before that *nox2*, the NADPH oxidase subunit of cytochrome b_{558} (formerly gp91^{phox}) and *nox1* were not present in breast cancer cells. While almost all cancer cells contained low levels of *nox4*, only the breast cancer cell line MDA-MB 436 expressed *nox3* message. A *nox5* short form was detected in MDA-MB 435 and MDA-MB-436 breast cancer cell lines and in the immortalized breast epithelial cell line HMEC184. Breast cancer cell lines were also tested for the presence of components of the neutrophil NADPH oxidase which form the active electron transfer complex in these cells. While we did not detect *p47*^{phox} or *p40*^{phox}, almost all of the cell lines showed high levels of *p22*^{phox}. This was expected since immunoblots had demonstrated that p22^{phox} is ubiquitously expressed in mammalian cells. It is very interesting in this respect that the only two cell lines without p22^{phox} in our screening are the breast epithelial cell line HMEC184 and MDA-MB 435. This has been confirmed by Western blot with specific anti-p22^{phox} antibodies. Our current results as described in Objective 2 would suggest that Nox4 might not be the ROS source, since Nox4 function is dependent on the co-expression of p22^{phox}. It is not clear from our data or published reports if Nox3 or Nox5 depend on complex formation with p22^{phox}. MDA-MB 436 contained not only p22^{phox}, but also the NADPH oxidase component p67^{phox}. Recently, two new homologs for p47^{phox} and p67^{phox} have been identified, termed p41 and p51 respectively (10-12). We have obtained the cDNAs and designed primers to investigate if these NADPH oxidase components are present in cancer cells and if they are required for Nox4 function.

We have obtained a new polyclonal anti-Nox4 antibody and were able to detect Nox4 in stable Nox4 HEK293 cell lines, in transiently transfected Cos cells and in cancer cells. We are in the process to use organelle markers to identify the Nox4 expressing cellular compartments, but it is already clear that Nox4 is predominantly located on internal membranes and vesicles, and not on the plasma membrane. A representative confocal image is shown below (Objective 2).

We conclude from these experiments that breast cancer cells contain Nox homologs and various components of the NADPH oxidase, but that their distribution and their ability to produce reactive oxygen species does not correlate directly with the presence of these proteins. It is obvious that we do not understand at this moment how Nox proteins are regulated and which Nox proteins are indicative of constitutive ROS generation. Cell-free assay systems reconstituted of cancer and normal epithelial cell fractions showed that ROS generation was independent of GTPγS or of neutrophil oxidase activators. We have therefore decided to focus on the two most abundant Nox isoforms (Nox4 and Nox5), since they seem to display a very different behavior when transfected into cells and to elucidate at first their function and regulation. These efforts may provide us with the necessary information to investigate then the regulation and function of Nox isoforms in cancer cells.

Objective 2: <u>Identification of signals regulating ROS production in cancer cells</u>

We investigated which intracellular signaling pathways or extracellular cues might enable cancer cells to produce ROS constitutively. Dominant negative as well as constitutively active signaling molecules, ranging from GTPases to signaling molecules involved in adhesion, were introduced into cancer cells. After several repetitions we concluded that none of these molecules abolished ROS generation in a significant manner. Starvation of cancer cells for 24-48 h suppressed ROS generation, which could be re-stimulated when starved cells were exposed to fresh serum. After 48 h the levels of ROS production reached the levels observed before starvation. This observation suggests that an autocrine loop or feedback mechanism exists which may be dependent on regulatory growth factors or hormones.

Since the identity of the ROS-generating enzyme in breast cancer cells is still under investigation, and the regulation of numerous Nox isoforms is unclear, we decided to evaluate Nox regulation in a more defined system than cancer cells with their multiple genetic changes. We prepared various cell lines stably expressing Nox4 and started to investigate Nox4 and Nox5 regulation by transient expression in non-transformed cell lines (HEK293, Cos). Nox4 was always constitutively active in untagged, wildtype form, while Nox5 did not release ROS into the medium (as measured with the HVA assay). Intracellular ROS production was measured with the nitroblue tetrazolium assay and showed that transfected Nox4 but not Nox5 produced ROS under these experimental conditions. We transfected also the Nox5 long form into HEK293 cells and observed basal as well as ionomycin-stimulated ROS generation. We decided to pursue the Nox4 isoform for our studies.

To evaluate how the presence of p22^{phox} affects Nox4-dependent ROS generation, we transfected Nox4 into two different Cos cell lines. One line has very low basal levels of p22^{phox}, while the other is stably transfected with p22^{phox}. The higher level of p22^{phox} protein increased ROS production by Nox4 about 30%, presumably through stabilizing Nox4 expression or by altering Nox4 localization. Another epithelial cell line, which lacks all identified Nox isoforms and p22^{phox}, showed that only transfection of a combination of Nox4 and p22^{phox} will generate ROS, while the both proteins alone were inactive. We performed co-localization experiments in several cell types and confirmed substantial co-localization of Nox4 and p22^{phox} on internal membranes. A representative confocal image is depicted below (Fig. 1). To substantiate further the requirement of p22^{phox} for Nox4 function, siRNA will be employed.

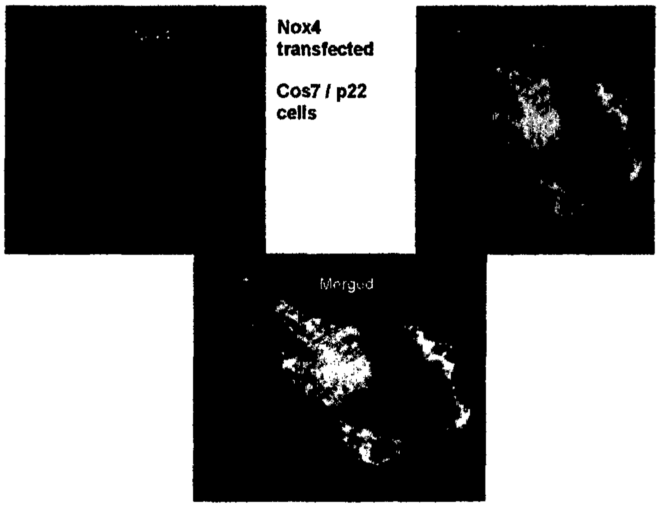

Fig.1: Confocal image of Nox4-p22^{phox} transfected Cos7 cells.

Objective 1: <u>Evaluation of ROS as second messengers in cancer cell metastasis</u>

We were not able to correlate the migratory behavior of different, ROS-producing breast cancer cell lines to the presence of Nox isoforms. We encountered also problems to assess migration and invasion of breast cancer cells with the *in vitro* assay systems commonly used. ROS generation in cancer cells was serum-dependent, which posed a problem for long-term migration, invasion and attachment studies, which are usually done without serum. To utilize a cleaner system, we established several Nox4- or vector control-expressing HEK293 cell lines by hygromycin selection. All seven Nox4-expressing clones produced ROS constitutively, while the control cells showed no production. Nox4-dependent ROS generation was also serum-dependent as observed with ROS production in cancer cells. Interestingly, we detected an increase in ROS generation by Nox4-expressing cells when plated on fibronectin instead of plastic, and an almost complete down-regulation of ROS generation upon detachment. Detachment was achieved by plating cells on matrices, which do not allow any cell attachment. We were also able to reattach suspension cells and to recover ROS generation by Nox4 upon attachment (Fig. 2). This seems to be an important regulatory mechanism for ROS production by Nox4 and our further studies will pursue this line of investigation. We are in the process of testing more components of integrin-mediated signaling for their influence on adhesion-dependent ROS production to identify the molecular mechanism

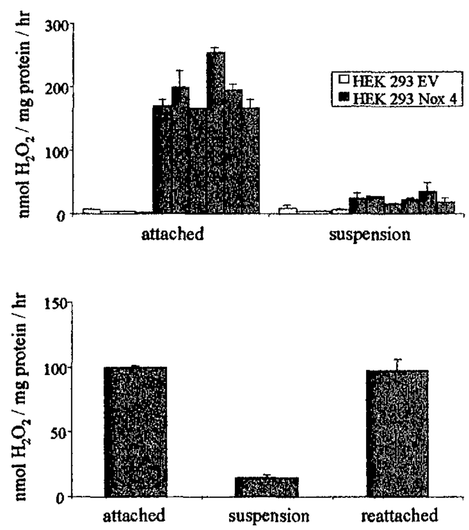

Fig. 2: ROS generation by Nox4-HEK293 cells in attached or suspended conditions.

behind this phenomenon. Important prerequisites of metastasis are not only cell movement or degradation of the extracellular matrix, but also stimulation of angiogenesis. Injection of NIH 3T3 fibroblasts stably expressing Nox1 into mice leads to tumor formation, which is characterized by highly vascularized tissue (6, 8). We tested HEK293 cells expressing the control vector or Nox4 for up-regulation of VEGF. VEGF message was up-regulated in Nox4 expressing cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Identification of *nox* genes and NADPH oxidase components in breast epithelial, breast cancer cells and control cells.
- Expression of various forms of Nox4 and Nox5 in epithelial cells to assess Nox isoform regulation and function.
- Identification of $p22^{phox}$ as essential partner for Nox4 function
- Analysis of stably transfected Nox4-expressing cell lines and control cell lines to evaluate parameters for cell adhesion, ROS generation and VEGF up-regulation.
- Discovery of adhesion as a crucial regulator of Nox4 function in stably transfected cells as well as in cancer cells.

REPORTABLE OUTCOMES:

Development of Nox4-expressing and control cell lines. We are in the process of preparing a manuscript describing the regulation of Nox4. The PI has submitted together with a collaborator an invited review detailing the regulation of Nox proteins (*Tibs*, submitted May 2003). The PI was co-organizer and invited speaker for a Banbury Conference (Cold Spring Harbor) on NADPH oxidases/Nox in November 2002.

CONCLUSIONS:

Several Nox family members have been detected in cancer cells, but also in immortalized breast epithelial cells. Their functions and regulation are still unknown, but are implicated in promoting cell growth, tumor formation in mice and possibly anti-bacterial defense (1, 6, 8, 9). Characterization of Nox protein regulation and subsequent functions in less complex systems will be necessary to shed light on their altered regulation in cancer cells. ROS have been implicated in tumor metastasis and angiogenesis in mice and the deregulated or altered state of Nox proteins in cancer cells may mediate these effects.

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